(C1 D4) D7

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: **0 080 806**
A1

(12) # EUROPEAN PATENT APPLICATION

(21) Application number: 82305632.0

(22) Date of filing: 22.10.82

(51) Int. Cl.³: C 12 N 15/00
A 61 K 39/02, C 12 P 21/00
C 12 N 1/00, C 12 N 9/10
//C12R1/19, A61K37/52, A61K7/16

(30) Priority: 22.10.81 US 314030

(43) Date of publication of application:
08.06.83 Bulletin 83/23

(84) Designated Contracting States:
AT BE CH DE FR GB IT LI LU NL SE

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(54) **Antigenic products of recombinant genes and related vaccines.**

(57) A vaccine for the immunization of a vertebrate comprising a non-pathogenic microbe that expresses a recombinant gene derived from an organism that is a pathogen of, or that produces an allergen of, said animal, where the gene codes for a gene product that is an antigen capable of being recognized by the immune system of the vertebrate and inducing antibody formation against the pathogen or allergen.

The use of microbes of the nature described above to produce non-living vaccines comprising such gene products essentially in the absence of other antigenic material from the parent organism.

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Antigenic gene products of recombinant genes, microbes containing such genes, vaccines containing the antigenic gene products or the microbes and a method of preparing such vaccines.

The invention described herein was made in the course of work supported by a grant from the Department of Health, Education, and Welfare, Public Health Service.

The present invention relates to vaccines and more particularly to antigenic gene products obtained from microbes containing one or more recombinant genes from a pathogenic organism as a vaccine against the pathogenic organism.

Bacterial infectious diseases are ubiquitous, although improved public health and the availability of antibiotics have decreased the incidence and minimized the consequences of infectious bacterial diseases in the world. In many underdeveloped countries, however, bacterial diseases are still rampant. Even in medically advanced countries, bacterial transposons that carry drug-resistance genes and numerous methods for dissemination of drug-resistance plasmids have resulted in drug resistance that has compromised the effectiveness of conventional methods of infectious disease control designed to counteract the effects of infection after infection has occurred. Thus, in the last several years, there has been increased emphasis on the development of vaccines that might prevent, if not eliminate, some infectious diseases.

Since the intact surface of a healthy epidermis is rarely, if ever, penetrated by bacteria, the point of initial infection is most often one of the mucous membranes. These include the conjunctiva and the oral, respiratory, gastrointestinal, and genitourinary surfaces. The mucous secretions of these membranes contain antibodies against invading pathogens and thus act as a first line of defense against invasive bacteria. Enhancing the immune response of the secretary system is thus a desirable goal in inducing immunity against pathogens.

Previously used vaccines against bacterial diseases have generally comprised (I) specific components purified from the etiologic agents, (II) the whole killed aetiologic agent, or (III) an avirulent derivative of the aetiologic agent as a live vaccine. Numerous vaccines of these three types exist, of which the following are selected examples:

U.S. 4,250,262, discloses methods for recovering the enzyme glucosyltransferase from Streptococcus mutans and the use of this purified enzyme in local immunization against dental caries, a Type I vaccine. Details for culturing the bacteria, purifying the enzyme, and using the enzyme to stimulate IgA antibody in saliva are presented for serotype a, c or g of S. mutans. Other examples of vaccines from purified specific components of bacteria are found in U.S. Patents 4,203,971 and 4,239,749, which disclose a vaccine useful against infection by Neisseria gonorrhoeae which consists of a glycoprotein from the outer coat material of gonococci. Injection of the glycoprotein stimulates a bactericidal antibody.

The use of dead S. mutans cells to immunize against tooth decay via administering in the mouth, which is disclosed in U.S. Patent 3,931,398, is an example of a

Type II vaccine. The inventors recognized that immunoglobulin A (IgA) antibodies were the antibodies being produced and that they resulted in a decrease in plaque formation.

A live bacterial vaccine (Type III) which contains selected strains of Escherichia coli bacteria is disclosed in U.S. Patent 3,975,517. The bacteria were treated with dilute formalin to attenuate or partially inactivate them before injection into the mammary gland of a sow. Antibody thereby produced was later found in the milk and protected newborn swine against E. coli infections. The formalin treatment that caused the E. coli inactivation was only a temporary attenuation of the bacteria and care had to be taken to prevent bacterial recovery before injection. Such recovery would have resulted in serious infection rather than protection.

Several problems exist in producing vaccines directly from pathogenic organisms, as indicated in the last example above. One principle obstacle to using whole bacteria (living or killed) or impure preparations obtained from the bacteria is the presence of many antigenic substances in such preparations that may cause undesirable cross reaction. For example, protein antigens produced by S. mutans have been reported to cross-react with antigens present in human heart muscle and thus, like other pathogenic bacterial proteins, may pose a problem of safety when used in a vaccine for humans.

Other problems arise in developing a vaccine capable of oral administration, certainly the most desirable form of administration when considered in terms of either widespread use by unskilled administrators in underdeveloped countries or in terms of patient comfort and acceptability. When oral administration is used to

stimulate an IgA response, the amount of material that is actually absorbed and capable of stimulating an effective immune response is usually low. The dose of antigen required for oral immunization generally far exceeds that required for systemic induction of immunity. It is assumed that a large portion of the antigen is degraded by enzymes of the gastrointestinal tract and may be eliminated or absorbed in a non-immunogenic form.

Accordingly, this invention enables the provision of a vaccine against bacterial infection that does not have antigenic material associated with it that is capable of causing undesirable antibody-antigen reactions in humans or animals.

The invention also enables the provision of a vaccine that does not have problems of cross-reactivity in a form suitable for oral administration.

Further, the invention enables the provision of a vaccine that will persist in the mucosal system of humans or animals and thereby stimulate the production of IgA in the secretory system.

According to a first aspect of the present invention there is provided a vaccine suitable for the immunization of a vertebrate, which vaccine comprises a non-pathogenic microbe that expresses a recombinant gene derived from a pathogen of a vertebrate to produce an antigen capable of inducing an immune response in the vertebrate against the pathogen. The microbe, which may be a bacterium, preferably belongs to a species that homes to a

lymphoepithelial structure of the vertebrate suitable microbes include members of the genera Escherichia (such as E. coli R DEC-1 and E. coli K-12) and Salmonella and Salmonella-Escherichia hybrids.

According to a second aspect of the present invention there is provided a vaccine suitable for the immunization of a vertebrate, which vaccine comprises an antigenic gene product of a gene derived from a first organism that is a pathogen of a vertebrate, wherein the gene was expressed in a second organism to produce the gene product and wherein the gene product is capable of inducing an immune response in the vertebrate against the pathogen. In this aspect of the invention, the vaccine is preferably essentially free of intact cells. The gene may code for a surface antigen or an enzyme, such as glycosyltransferase of the pathogen, which may belong to one of the following species: Escherichia coli, Vibrio cholerae, Shigella, Pseudomonas aeruginosa, Neisseria gonorrhoeae, Chlamydia trachomatis and, particularly, Streptococcus mutans.

According to a third aspect of the present invention, there is provided a method of producing a vaccine suitable for the immunization of a vertebrate against a pathogen, comprising the steps of selecting a gene coding for an antigen in a pathogenic organism and inserting the gene into a carrier microbe, wherein the carrier microbe expresses the gene to produce a gene product capable of inducing antibodies against the pathogenic organism or a metabolic product of the pathogenic organism when the gene product is administered to the vertebrate. A vaccine produced according to this method may comprise the gene product in the presence or absence of the carrier microbe, which is preferably non-pathogenic. The gene may be inserted into a plasmid prior to insertion into the carrier microbe.

According to a fourth aspect

of the present invention there is provided a vaccine suitable for the desensitization of a vertebrate to an allergen, which vaccine comprises an antigenic gene product of a gene derived from a first organism that produces an allergen of said vertebrate, wherein the gene is expressed in a second organism which produces the gene product and wherein the gene product is an allergen of said vertebrate. It will be appreciated that the present invention enables the achievement of a method for stimulating the immune system in a vertebrate, which method comprises the step of administering to a vertebrate a non-pathogenic microbe that expresses a recombinant gene derived from a pathogen of the vertebrate to produce an antigen capable of inducing an immune response in the vertebrate against the pathogen. The invention also enables the achievement of a method of desensitizing a vertebrate to an allergen, which method comprises the step of administering to the vertebrate a non-pathogenic microbe that expresses a recombinant gene derived from an animal that produces an allergen of the vertebrate

According to a fifth aspect of the present invention, there is provided a non-pathogenic microbe that expresses a recombinant gene derived from a pathogen of a vertebrate to produce an antigen capable of inducing an immune response in the vertebrate against the pathogen.

According to a sixth aspect of the present invention, there is provided an antigenic gene product of a gene derived from a first organism that is a human pathogen wherein the gene was expressed in a second

organism to produce the gene product and wherein the gene product is capable of inducing an immune response in a human against the pathogen.

A more complete appreciation of the invention and many of the attendant advantages thereof will be readily obtained as the invention becomes better understood by reference to the following exemplary detailed description when considered in connection with the accompanying drawings, wherein:

FIGURE 1 shows an Ouchterlony analysis of reactions between antiserum to purified spaA protein and concentrated culture supernatuant fluids from strains of S. mutans serotypes a,c,e,f,g and d;

FIGURE 2 shows an Ouchterlony analysis of antisera to purified spaA protein (a spaA) and S. mutans serotype c antigen I/II (a1/II) with purified spaA protein, an extract from E. coli χ1274 (pYA727) which produces spaA protein and an extract from E. coli χ1274 which contains the cloning vector (pACYC184);

FIGURE 3 shows radiolabelled proteins synthesized in minicells obtained from (1) χ1849 (pBR322), (2) χ1849, (3) χ1849 (pYA601), where the 55k protein is glucosyltransferase and bla=β-lactamase;

FIGURE 4 shows a physical map of pYA601, with the S. mutans DNA insert consisting of two HindIII restriction fragments, A and B, of 1360 and 370 bp in length, respectively; and

FIGURE 5 shows an Ouchterlony analysis of spaA protein present in the periplasm (B) and cytoplasm (C) of E. coli HB101 (pYA721) where well A contains anti-spaA serum.

Microorganisms prepared by the genetic engineering procedures described herein and suitable pathogenic bacteria from which genes may be obtained are exemplified by cultures now on deposit with the American Type Culture collection, 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A.. These cultures are identified as follows:

Escherichia coli 1274 (pYA619) (ATCC No. 31984), Escherichia coli HB101 (pYA726) (ATCC No. 31985), and Escherichia coli 1274 (pYA601) (ATCC No. 31986) strains derived by genetic transfer thereto, and containment therein, of genes from Streptococcus mutans; and

Streptococcus mutans UAD90 (ATCC No. 31987), Streptococcus mutans UAD50 (ATCC No. 31988), and Streptococcus mutans UAD308 (ATCC No. 31989) donor strains.

A sub-culture of each of these strains can be obtained from the collection of the American Type Culture Collection at the above-given address.

The present inventor has determined that many of the problems associated with previous vaccines against pathogens can be minimized by using non-pathogenic microbes to produce selected antigens from pathogens using recombinant DNA techniques, thus providing these antigens in a form essentially free of contaminating antigens from the pathogenic organism. It is preferred to introduce the vaccine directly to the mucous membranes to stimulate IgA production. Recent studies have shown that a central pathway for the antigenic stimulation of IgA precursor cells exists in gut-associated lymphoid tissues (GALT) and bronchial-associated lymphoid tissues (BALT) and is followed by dissemination of sensitized cells to distant mucosal sites. Available data indicate

that the lamina propria of the gut and respiratory tract, mammary and salivary glands, as well as the genito-urinary tract, are supplied by sensitized cells from GALT and BALT. For example, lymphocytes are spread throughout the inner layer of the intestine as isolated cells or small cell clusters. Some of the cell clusters develop into distinct organs, known as lymphoepithelial structures. The principal ones in humans are (1) tonsils (in the pharyngeal wall), (2) the appendix (at the junction of the small and large intestine, and (3) Peyer's patches (oblong lymphoid cell clusters found mostly at the terminal portion of the small intestine). For example, Weisz-Carrington et al, J. Immunol. 123, 1705 (1979), have recently shown that oral immunization with ferritin results in the appearance of antibody-producing cells in extra-intestinal lymphoid tissues of mice. Furthermore, in secretory glands, the immunoglobulin class of cells producing anti-ferritin was mostly IgA, while in spleen or peripheral lymph nodes, IgM and IgG ferritin-binding cells were encountered. It is assumed that the major natural pathway for stimulation of the immune system occurs through GALT and BALT sensitization. The schematic representation that follows shows pathways of sensitization of B and T lymphocytes, their emigration from GALT and BALT, homing to distant mucosal tissues and differentiation into IgA-producing plasma cells.

SCHEME I

THE COMMON MUCOSAL IMMUNE SYSTEM

Ingested or Inhaled Antigens

↓

Antigen penetration via specialized mucosal epithelial cells to T and B lymphocytes in gut or bronchial-associated-lymphoid tissue (GALT or BALT)

↓

Local Sensitization of T and B cells

↓

Migration of T and B Cells to Mesenteric Lymph Nodes

↓

Thoracic Duct Lymph

↓

Blood Circulation

↓

Homing of B- and T-cells to distant mucosal tissues

↓	↓	↓	↓	↓	↓
Lamina Propria of gut and Respiratory Tract	Mammary Glands	Salivary Glands	Lacrymal Glands	Genitourinary Tissues	Others

↓

Differentiation and clonal expansion to IgA Producing cells (in the presence of antigen)

In a preferred embodiment of the invention, carrier bacteria can be used to deliver selected antigens to the GALT, for example to the Peyer's patches of the ileum. Some genera of baceria, such as Salmonella, are known to

home to the Peyer's patches. In a well-studied case with E. coli RDEC-1, the bacteria specifically attach to, invade and persist in the Peyer's patches of the ileum of rabbits, for which this bacterium is species specific. Other E. coli strains specific to the human species are know to colonize Peyer's patches in humans. S. typhimurium-E. coli hybrids have also been shown to colonize Peyer's patches in humans. If these carrier bacteria contain and express a recombinant gene from a pathogenic organism, antibodies against the pathogen will be induced. With the advent of recombinant DNA techniques, it now becomes possible to develop totally unique vaccines in which specific antigens are produced, not by the aetiologic agent, but by another host strain of bacteria capable of expressing the gene for that antigen. It is also possible, when antigens might cross-react with an antigen of the mammalian host and thus potentiate the induction of autoimmunity, to use recombinant DNA techniques to alter the gene so that the affecting cross-reacting antigenic determinant is not produced. Thus, recombinant DNA techniques can be employed to develop vaccines that do not have any material capable of cross-reacting with mammalian host antigens or capable of eliciting an autoimmune state.

It is apparent that the present invention has wide applicability to the development of effective vaccines against bacterial or viral disease agents where local immunity is important and might be a first line of defense. Some examples are vaccines for the control of enteric diseases caused by enteropathogenic E. coli strains, Vibrio cholerae, Salmonella typhi, and Shigella species. Other vaccines of the invention could be used in the control of persistent Pseudomonas aeruginosa infections in patients with cystic fibrosis, in protection against pneumonic plague caused by Yersinia

pestis, and in prevention of E. coli urinary tract infections and of Neisseria gonorrhoeae, Treponema pallidum, and Chlamydia trachomatis-caused venereal diseases as well as Chlamdi caused eye infections. Species of Streptococci from both group A and group B, such as those species that cause sore throat or heat diseases are additional examples of bacteria within the scope of this invention from which genes could be obtained. Viral vaccines, such as those produced against influenza viruses, are also encompassed by this invention. Viral vaccines can also be produced against other viruses, either DNA or RNA viruses, for example from the classes Papovirus, Adenovirus, Herpesvirus, Poxvirus, Parvirus, Reovirus, Picornavirus, Myxovirus, Paramyxovirus, or Retrovirus.

In one of its embodiments, the invention can be described as a vaccine for the immunization of a vertebrate animal comprising a live non-pathogenic microbe that expresses a recombinant gene derived from an organism that is a pathogen of or that produces an antigen of said animal. Each of the terms in this embodiment of the invention is analyzed in the following discussion.

By vaccine is meant an agent used to stimulate the immune system of a living organism so that protection against future harm is provided. Immunization refers to the process of inducing a continuing high antibody level in an organism, which is directed against a pathogen or antigen to which the organism has been previously exposed. Although the phrase "immune system" can encompass responses of unicellular organisms to the presence of foreign bodies, e.g., interferon production, in this application the phrase is restricted to the anatomical features and mechanisms by which a multi-cellular organism produces antibodies against an

antigenic material which invades the cells of the organism or the extra-cellular fluid of the organism. The antibody so produced may belong to any of the immunological classes, such as immunoglobulins A, D, E, G, or M. Of particular interest are vaccines which stimulate production of immunoglobulin A (IgA) since this is the principle immunoglobulin produced by the secretory system of warm-blooded animals, although vaccines of the invention are not limited to those which stimulate IgA production. For example, vaccines of the nature described herein are likely to produce a broad range of other immune responses in addition to IgA formation, for example, cellular and humoral immunity. Immune response to antigens is well studied and widely reported. A survey of immunology is given in Davis, Dulbecco et al, Microbiology: Immunology and Moelcular Genetics, Third Edition, Harper and Row, Hagerstown Md, USA (1980), the entire of which is herein incorporated by reference.

A vertebrate is any member of the subphylum Vertebrata, a primary division of the phylum Chordata that includes the fishes, amphibians, reptiles, birds, and mammals, all of which are characterized by a segmented bony or cartilaginous spinal column. All vertebrates have a functional immune system and respond to antigens by producing antibodies. Thus all vertebrates are capable of responding to vaccines. Although vaccines are most commonly given to mammals, such as humans or dogs (rabies vaccine), vaccines for commerically raised vertebrates of other classes, such as the fishes, if of the nature described herein, are within the scope of the present invention.

One of the essential features of this embodiment of the invention is the use of a non-pathogenic microbe as a carrier of the gene product which is used for stimulating

antibody response against a pathogen or allergin. Non-pathogenic does not mean that a microbe of that genus or species can not ever function as a pathogen, but that the particular microbe being used is not a pathogen for the particular animal being treated. The microbe may belong to a genus or even a species that is normally pathogenic but must belong to a strain that is non-pathogenic. By pathogenic is meant capable of causing disease or impairing normal physiological functioning. Microbes include viruses, bacteria, protozoa, and unicelluar fungi. It is also possible to use synthetic viruses as carriers comprising viral coat proteins assembled in a test tube with non-viral DNA.

Techniques for transferring genetic material from one organism to another have recently become widely available as the result of rapidly expanding recombinant DNA technology. In this application, genetic material that has been transferred from one organism into a second in such a manner that reproduction of the second organism gives rise to descendents containing the same genetic material is referred to as a recombinant gene. The term gene is being used here in its broadest sense to represent any biological unit of heredity. It is not necessary that the recombinant gene be a complete gene as present in the parent organism, which was capable of producing or regulating the production of a macromolecule, for example, a functioning polypeptide. It is only necessary that the gene be capable of serving as the template used as a guide in the production of an antigenic product. The product may be one that was not found in that exact form in the parent organism. For example, a functional gene coding for a polypeptide comprising 100 amino acid residues may be transferred in part into a carrier microbe so that a peptide comprising only 75, or even 10, amino acid residues is produced by

the cellular mechanism of the host cell. However, if this gene product is an antigen that will cause formation of antibodies against a similar antigen present in the parent organism, the gene is considered to be within the scope of the term gene as defined in the present invention. At the other end of the spectrum is a long section of DNA coding for several gene products, one or all of which can be antigenic. Thus a gene as defined and claimed here is any unit of heredity capable of producing an antigen. The gene may be of chromosomal, plasmid, or viral origin.

In order for the gene to be effective in eliciting an immune response, the gene must be expressed. Expression of a gene means that the information inherent in the structure of the gene (the sequence of DNA bases) is transformed into a physical product in the form of a polypeptide, RNA molecule, or other biological molecule by the biochemical mechanisms of the cell in which the gene is located. These may be the cellular mechanisms of the carrier microbe if the microbe is a bacterium protozoan, or yeast, or the cellular mechanisms of an infected cell if the microbe is a virus. The biological molecule so produced is called the gene product. The term gene product as used here refers to any biological product or products produced as a result of the biochemical reactions that occur under the control of a gene. The gene product may be, for example, an RNA molecule, a peptide, or a product produced under the control of an enzyme or other molecule that is the initial product of the gene, i.e., a metabolic product. For example, a gene may first control the synthesis of an RNA molecule which is translated by the action of ribosomes into an enzyme which controls the formation of glycans in the environment external to the original cell in which the gene was found. The RNA molecule, the

enzyme, and the glycan are all gene products as the term is used here. Any of these as well as many other types of gene products, such as glycoproteins, will act as antigens if introduced into the immune system of an animal. Protein gene products, including glycoproteins, are preferred gene products for use as antigens in vaccines.

In order for a vaccine to be effective in producing antibodies, the antigenic material must be released in such a way that the antibody-producing mechanism of the vaccinated animal can come into play. Therefore the microbe carrier of the gene product must be introduced into the animal. In order to stimulate a preferred response of the BALT and GALT cells as discussed previously, introduction of the microbe or gene product directly into the gut or bonchus is preferred, although other methods of administering the vaccine, such as intramuscular or subcutaneous injection or intrapenial or vaginal administration, are possible.

If a carrier microbe is used, once the carrier microbe is present in the animal, the antigen needs to become available to the animal's immunological system. This may be accomplished when the carrier microbe dies so that the antigen molecules are released. However, it is preferred that a gene be selected that controls the production of an antigen that will be made available by the carrier cell to the outside environment without the death of the cell. In this way it is possible to use a viable microbe that will persist in the vaccinated animal, for example in its Peyer's patches, and continue to produce antigen, thereby continually inducing antibody formation. A preferred gene product under these circumstances in a product that is transferred through the cell membrane into the external environment or a product that becomes attached to or embedded in the

external membrane so that all or part of the gene product is exposed to the environment. Typical of this latter type of gene product are antigens normally found on the surface of the organism against which protection is desired. If these antigens are transported to the cell surface in a normal manner, antibody formation against the antigens will be greatly enhanced.

However, it is also possible to use a non-viable carrier that dies and releases cytoplasmic antigens. When non-viable carrier microbes are used, it is possible to employ a wider range of carrier microbes, for example bacteria that would normally be pathogenic, than when viable carriers are used. The use of pathogens to deliver antigens from other pathogens to the GALT or BALT would be inappropriate if it were not for the fact that such pathogens can be rendered "avirulent" while retaining ability to invade Peyer's patches. Alteration of E. coli strains to preclude their survival in nature is well-known, and has been described in, for example, U.S. Patent 4,190,495, which is herein incorporated by reference. The same deletion (Δ) mutations (e.g., ΔthyA) used in the laboratory to create bacteria that require a particular nutrient can be used to preclude long-term survival without preventing penetration of Peyer's patches. The Δasd mutation, which eliminates aspartic acid semialdehyde dehydrogenase and thus imposes a requirement for diaminopimelic acid (DAP), would also result in DAP-less death in vivo with release of intracellular and periplasmic proteins. Furthermore, deletion mutations in S. typhimurium that prevent enterochelin synthesis result in strains that are avirulent but which can be used to induce effective immunity against subsequent Salmonella infection. This bacterium is described in Hoiseth and Stocker, Nature 291, 238 (1981), which is herein incorporated by

reference. . Such a bacterium provides a convenient source of carrier microbes.

The organism from which the recombinant gene is derived may be any pathogen of the animal being vaccinated or may be an organism that produces an allergen or other antigen of the animal. Allergens are substances that cause allergic reaction, in this case in the animal which will be vaccinated against them. Many different materials may be allergens, such as animal hair and pollen, and the allergic reaction of individual animals will vary for any particular allergen. It is possible to induce tolerence to an allergen in an animal that normally shows an allergic response. The methods of inducing tolerence are well-known and generally comprise administering the allergen to the animal in increasing dosages. Further discussion of tolerence induction is given in the Davis, Dulbecco et al publication previously cited.

Administration of a live vaccine of the type disclosed above to an animal may be by any known or standard technique. These include oral ingestion, intestinal intubation, or broncho-nasal spraying. All of these methods allow the live vaccine to easily reach the BALT or GALT cells and induce antibody formation and are the preferred methods of administration. Other methods of administration, such as intravenous injection, that allow the carrier microbe to reach the animal's blood stream may be acceptable under unusual circumstances but are ordinarily to be avoided in order to prevent systemic infection by the carrier microbe. Intravenous injection is acceptable, however, with other embodiments of the invention using purified antigens, as is described later.

Since preferred methods of administration are oral ingestion and intestinal intubation, preferred carrier microbes are those that belong to species that home

preferentially to any of the lymphoepithelial structures of the intestines of the animal being vaccinated. These strains are preferred to be non-pathogenic derivatives of enteropathogenic strains produced by genetic manipulation of enteropathogenic strains. Strains that home to Peyer's patches and thus directly stimulate production of IgA are most preferred. In humans these include strains of E. coli, Salmonella, and Salmonella-E. coli hybrids that home to the Peyer's patches.

Recombinant DNA techniques are now sufficiently well known and widespread so as to be considered routine. In very general and broad terms, this method consists of transferring the genetic material, or more usually part of the genetic material, of one organism into a second organism so that the transferred genetic material becomes a permanent part of (recombines with) the genetic material of the organims to which it is transferred. This usually consists of first obtaining a small piece of DNA from the parent organism either from a plasmid or a parent chromosome. A plasmid (also called an extrachromosemal element) is a hereditary unit that is physically separate from the chromosome of the cell. The DNA may be of any size and is often obtained by the action of a restriction endonuclease enzyme which acts to split DNA molecules at specific base-pair sites. The DNA pieces may be transferred into a host cell by various means such as transformation (uptake of naked DNA from the external environment, which can be artificially induced by the presence of various chemical agents, such as calcium ions), conjugation (sexual reproduction of bacteria), and transfection (transfer of non-viral DNA by phages or in the presence of phage coat proteins). Other methods such as tranduction are also suitable. Once the parent DNA is in the carrier cell, it may continue to exist as a separate piece (generally true of complete

transmitted plasmids) or it may insert into the host cell chromosome and be reproduced with the chromosome during cell division.

Although transferring genetic material is relatively straightforward, predicting which transfers will result in expressed genes is not yet possible. This selection process, however, does not present any difficulty to the present invention. Since the host microbe must express the transferred gene and thereby produce an antigen, a "shotgun" approach works well. Antibodies are first produced against the desired antigen, for example, fragments of pathogenic cell membranes, by standard techniques. DNA from the organism that is the source of the antigen is cleaved into multiple fragments by endonucleases, and the fragments are inserted randomly into carrier microbes. The microbes that express antigens from the pathogen can be easily identified by their reaction with labelled antibody against pathogen antigens. Antigen-expressing microbes can be selected and cloned to give the desired recombinant organism. Shotgun cloning is well known and is described in detail in Sinsheimer, Ann, Rev. Biochem. 46, 415 (1977), which is herein incorporated by reference.

The techniques of gene transfer are not considered to be part of this invention, and any method capable of producing recombinant organisms comprising genes from pathogenic organisms that are expressed in non-pathogenic microbes will suffice. The techniques of DNA isolation, gene cloning, and related techniques are disclosed in great detail in, for example, Recombinant DNA, Methods of Enzymology, Volume 68, Ray Wu, ed., Academic Press (1979), which is herein incorporated by reference.

It is also possible in a second and broader embodiment broader of this invention to provide a vaccine

for the immunization of a vertebrate comprising a gene product obtained from a microbe that expresses a recombinant gene derived from an organism that is a pathogen of said vertebrate wherein said gene product is capable of producing an immune response in said vertebrate against said pathogen.

A gene product so produced will be free of other antigenic material derived from the organism against which immunization or tolerence is being induced. This is of particular importance when dealing with pathogenic organism is known to induce antibodies which react with various cells or organs of the infected animal. For example, some protein antigens produced by S. mutans have been reported to cross-react with antigens present in human heart muscle, and thus, like other streptococcal proteins, may pose a particular problem of safety when used in a vaccine for humans. Such problems can be avoided by selecting clones of recombinant microbes that produces only antigens that do not exhibit dangerous cross-reactivity.

The principle difference between the two embodiments discussed is that in the first embodiment the living microbes serve as carriers of the antigen while in the second embodiment the vaccine comprises the antigenic gene product in the absence of the living microbes that produce it. Several advantages arise as a result of this second embodiment. Principally, there is no danger of uncontrolled infection by the carrier microbe. Although there was little danger of uncontrolled infection in the first embodiment because of the control available by choice of non-pathogenic strains of microbes and the ability to produce non-viable strains, it was nevertheless necessary to use the microbe that produced the antigenic gene product directly as the vaccine. In the second embodiment, it is possible to use any organism

into which genes from the pathogen or antigen-producing organism can be introduced to manufacture the gene product essentially in the absence of other antigens capable of harming the vaccinated animal. Thus, an organism that gives a high yield of the desired gene product may be grown and harvested and the gene product may be separated and used as a vaccine without any need for making the organism acceptable for internal administration. A high-yielding non-compatable organism can therefore be used to produce the vaccine.

Any antigenic gene product, for example, those previously discussed, may be produced in accordance with the second embodiment. Shotgun cloning and selection of clones by antibody reaction works equally well for the second embodiment.

Separation of the antigen from other materials present in the second organism remains to be accomplished after clone selection and growth. The separation of specific components from complex mixtures by their physical and chemical properties is well developed. No more than routine experimentation is required for the separation of most antigenic gene products, since the presence of these gene products in any fraction obtained from a mixture can be determined easily by assaying for antibody/antigen reations. One suitable method of separation that should be applicable to separations of all antigens is the use of an affinity chromotagraphy column comprising an antibody bound to a support material. Such an affinity column should retain the antigen on the column when a crude preparation containing the antigen is passed over the column. Since shotgun cloning and selection of clones by antibody reaction is a preferred method of selecting suitable clones, antibodies will already exist for desired antigens, and preparation of a suitable affinity chromotagraphy column should not

be difficult. Other methods of purification, such as ammonium sulphate precipitation and gel filtration, are also suitable.

In summary, the vaccines of this invention may be produced by genetic engineering of microbes, comprising two essential steps:

a) selecting a gene coding for an antigen in a pathogenic organism;

b) inserting the gene into a non-pathogenic carrier microbe, wherein the microbe expresses the gene to produce a gene product capable of inducing antibodies against the pathogenic organism or its metabolic products when the gene product (either in the presence or absence of the carrier microbe) is administered to a vertebrate which is to be protected against the pathogenic organism by the vaccine. Steps which take place after these two steps, for example, preparation of the microbe culture for administration or purification of the antigenic gene product are not considered part of the present invention, but are merely routine technical steps in the preparation of the vaccine into its final form. Nevertheless, methods of carrying out the later steps are summarized below.

Vaccines of this invention may contain either the carrier microbe capable of expressing the pathogen derived gene or may contain the gene product in a cell- and viral-free form. The method of administration is preferably varied to fit the type of vaccine being used. Vaccines containing carrier microbes are preferably administered orally, particularly when colonization of Peyer's patches is desired. Dosage will vary depending on the ability of the carrier microbe to express the recombinant gene and thus produce antigen, but will generally be in the 1-10 ml range at

concentrations ranging from about 10^2 to 10^7 microbes per ml. Preferred oral preparations are enteric-coated. Such preparations are resistant to acid and enzymes in the stomach of the innoculated animal while dissolving in the intestines. Various enteric-coating are known in the art, for example as disclosed in U.S. Patent Nos. 3,241,520 and 3,253,944, and are commerically available. A method suitable for preparation of enteric-coated capsules is described in U.S. Patent 4,152.415, which is herein incorporated by reference, and can be easily modified to provide capsules containing the carrier microbes of the present invention. The only required modification is adjustment of the moisture content of the antigen-containing slurry to prevent excessive drying of viable microbes. The amount of drying required is easily determined by routine experimentation.

Cell- and viral-free vaccines containing the pathogen-derived gene product may also be administered orally as described above or may be administered parenterally (e.g., by intramuscular, subcutaneous, or intravenous injection). The amount required will vary with the antigenicity of the gene product and need only be an amount sufficient to induce an immune response typical of existing vaccines. Routine experimentation will easily establish the required amount. Typical initial dosages of vaccine could be 0.001-1 mg antigen/kg body weight, with increasing amounts or multiple dosages used as needed to provide the desired level of protecton.

The pharmaceutical carrier in which the vaccine is suspended or dissolved may be any solvent or solid that is non-toxic to the innoculated animal and compatable with the carrier organism or antigenic gene product Suitable pharmaceutical carriers include liquid carriers, such as normal saline and other non-toxic salts at or

near physiological concentrations, and solid carriers, such as talc or sucrose. Adjuvants, such as Freund's adjuvant, complete or incomplete, may be added to enhance the antigenicity of the gene product if desired. When used for administering via the broncial tubes, the vaccine is suitably present in the form of an aerosol.

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific example which is provided herein for purposes of illustration only and is not intended to be limiting unless otherwise specified.

Example

DNA from S. mutans strains 6715 (serotype g, 45% guanine + cytosine content) and PS14 and GS-5 (serotype c, 35% guanine + cytosine) was cloned into suitable strains of E. coli K-12. Shotgun cloning experiments were performed to determine whether S. mutans genes were expressed in E. coli and, if so, whether they would complement E. coli gene defects. The DNA was isolated from the S. mutans strains UAB50, UAB90, and UAB308 by treating the S. mutans cells with the enzyme mutanolysin and then lysing the bacteria with the detergent sodium dodecyl sulphate.. The DNA was recovered by ethanol precipitation, restricted with various restriction endoneculeases such as EcoRI, HindIII, BamHI, and PstI and used to anneal to pBR322 or pACYC184 vectors cut with the homologous enzyme. Recombinant molecules were formed by the addition of polynucleotide joining enzyme (or DNA ligase) and suitable strains of E. coli K-12 such as HB101, χ1274, and χ1849 were transformed by the calcium chloride cold shock method. In other experiments, recombinant molecules were formed by ligating DNA to the cosmid vector pJC74, packaging the recombinant molecules by in vitro packaging methodology with components to

introduce the recombinant cosmid DNA into suitable strains of E. coli K-12 such as HB101 lysogenic for the thermo-inducible lambda prophage λ cI857. Transformant or transfectant clones were selected for resistance to an antibiotic for which the cloning vector carried the appropriate drug resistance gene. Tests using a variety of multiple mutant E. coli strains indicated that about 40% of the tested E. coli gene defects for purine, pyrimidine and amino acid biosynthesis and carbohydrate use could be complemented by S. mutans genetic information. The presence of S. mutans DNA was verified using Southern blotting analysis. E. coli deletion mutants lacking a given function would sometimes grow as rapidly with S. mutans genetic information cloned on the multicopy plasmids pBR322 and pACYC184 (available commerically from Bethesda Research Laboratory, Rockville, Md. USA) as they would if provided with the optimal amount of the required supplement. In general, most S. mutans genes were expressed constitutively and were not subject to repression by end products or inducible by appropriate substrates. One exception was a cluster of four or five S. mutans genes for galactose utilization. These genes were coordinately regulated and moderately inducible in E. coli in exactly the same manner as they were in S. mutans. This suggested some type of autogenous regulation, an hypothesis currently being tested. Gene products of S. mutans that are necessary for the transport and phosphorylation of sugars and that probably associate with the cytoplasmic membrance of S. mutans function in E. coli in much the same way. S. mutans gene products that are normally on the cell surface of S. mutans or are excreted into the growth medium were transported across the E. coli cytoplasmic membrane and ended up in the periplasmic space. E. coli perA mutants that are defective in

transport of various periplasmic proteins from the cytoplasm into the periplasm were still able to transport certain S. mutans cell surface gene products into the periplasmic space in E. coli .

A goal in cloning S. mutans genes in E. coli was to identify those genes that might contribute to the ability of S. mutans to colonize the tooth surface. Glucosyltransferase cleaves sucrose to yield fructose while polymerizing glucose into glucans. S. mutans synthesizes both water-soluble glucans which have α 1→6 linkages and water-insoluble glucans which are branched and have α 1→3 linkages in addition to the α 1→6 linkages. A gene for glucosyltransferase activity from both S. mutans PS14 and GS-5 was cloned into E. coli. This gene in pYA601 (from PS14) and pYA619 (from GS-5) is contained on a 1730 bp DNA fragment which encodes an enzyme that synthesizes water-soluble glucans. This glucosyltransferase, which has a 55,000 molecular weight, is transported into the E. coli periplasm without modification. There it is able to cleave sucrose (which can get into E. coli's periplasmic space) to liberate fructose which can be used for growth and to synthesize glucans. As previously mentioned, immunization with purified glucosyltansferase conferred protective immunity against S. mutans induced dental caries.

In order to successfully clone various S. mutans genes for cell-surface associated proteins that might not have enzymatic activity, an immunological screening procedure was used. In this approach, antibodies were raised against ammonium sulphate precipitated extracellular proteins of S. mutans 6715. The pJC74 cosmid cloning system was used and the recombinant molecules were introduced into an E. coli strain lysogenic for the λcI857 thermosensitive prophage. This cloning system is described in Collins et al, PNAS, 75,

4242 (1978), which is herein incorporated by reference. Induction of lysogenic cells in colonies grown at 30°C by shifting the temperature to 37°C caused cell lysis to release any antigens, even if they were localized to the cytoplasm rather than being transported to the exterior surface of E. coli. Using this method and the formation of precipitin bands around the colonies containing antigens cross-reacting with the antibodies against S. mutans extracellular proteins, it was possible to identify numerous clones which expressed S. mutans cell surface associated protein antigens. These clones were given the identifications pYA701 through pYA721. One clone (pYA721) was of particular interest. This clone specifies a 210 k protein (the spaA protein) which was orginally encoded by a 5.5 Mdal fragment of S. mutans DNA. This particular protein antigen cross-reacts with protein antigens in all of the serotypes of S. mutans except for serotype b which is a group of S. mutans (corrrectly known as S. ferrus) that principally colonizes rodents and not humans. An Ouchterlony analysis of this cross-reaction is shown in Fig. 1.

It should be noted that this 210 k protein from serotype g S. mutans is immunologically related to the antigen I/II (Fig. 2) of Russell who found that injection of this protein purified from the serotype c S. mutans strain Ingbritt into Rhesus monkeys was protective against S. mutans induced dental caries. (Russell et al., Immunol, 40, 97 (1980)).

Immunization of the submandibular region of gnotobiotic rats with killed S. mutans cells induces salivary agglutinins against S. mutans which are in the IgA class. Such immunization was protective against subsequent challenge with virulent S. mutans strains in that mean caries scores were significantly lower in the immunized rats than in the control group that had not

been immunized. Subsequent studies demonstrated that ingestion of killed S. mutans cells could likewise stimulate salivary secretory IgA against S. mutans and also confer protective immunity when rats were subsequently challenged with virulent S. mutans strains. These results have been confirmed in human studies in which it was shown that oral ingestion of killed S. mutans resulted in production of sIgA in tears and salvia. These antibodies were specific against the S. mutans vaccine strain. These studies also indicated a negligible increase in serum agglutinins and gave no evidence of stimulation of any human heart reactive antibodies.

The two S. mutans gene products expressed in E. coli that have been most intensely studied have been the spaA protein and glucosyltransferase (GTF).

Glucosyltransferase can be assayed by its ability to hydrolyze sucrose to liberate reducing sugars. The Somogyi reagent is used and the reaction is followed at 520 nm. This assay can be used on extracts and on toluenized cells. Enzyme activity in non-denaturing polyacrylamide gels can be measured by soaking the gels in 0.1 M sucrose (in 0.2 M phosphate buffer, pH 7.0) for 1 to 2 h followed by treating with triphenyl tetrazolium chloride in alkali in the dark to identify the band(s) containing reducing sugar generating activity. It is also possible to assay the GTF for synthesis of glucan using uniformly labelled radioactive sucrose but this is more costly and time consuming. Monospecific rabbit antibody against GTF produced by E. coli and monoclonal antibodies can be used for radioimmunoassay (RIA) and the antibody tagged with fluorescein or rhodamine isothiocyanate can be used to identify bacterial cells that have GTF on their cell surface.

In terms of purification, GTF is a 55 k protein (Fig. 3) which is soluble in 33% ammonium sulfate. Many E. coli proteins are thus removed from the cell extract by ammonium sulfate precipitation. This is followed by a combination of chromatography on diethylaminoethyl (DEAE) cellulose (DE52; Whatman Ltd.), Ultrogel AcA54 and Ultrogel AcA44 (LKB) with concentration of pools by ultrafiltration with Amicon membranes to result in a homogeneous GTF preparation. Protein contaminants are less than a few percent since other protein bands are undetectable by either radioactive labelling and radioautography or by Coummassie blue straining of SDS polyacrylamide gels. Using this method about 50% GTF recovery can be achieved and some 10 to 15 mg of GTF from a one litre culture E. coli cells harbouring the pYA601 plasmid have been obtained.

GTF is synthesized constitutively in E. colix1849. E. coli cells growing on glucose or raffinose with isopropyl-thio-β-galactopyranoside (IPTG) produce about 50% as many glucosyltransferase molecules as β-lactamase molecules which are also encoded on the pBR322 cloning vector (Fig. 3). The gene for glucosyltransferase spans two HindIII fragments of 1360 and 370 bp (Fig. 4). The larger fragment contains the RNA polymerase binding site. (Both fragments have also been cloned within a BamHI fragment.) If one subtracts 100 bp for promoter and terminator control sequences, then the two HindIII fragments would specify a protein of only 55 to 58 k in molecular weight.

Because of its size, the spa A protein can be purified in several steps. When the protein is purified from S. mutans, the cell-free culture supernatant fluid is concentrated to 5% of its original volume by ultrafiltration, sucrose is added to a concentration of 10% (w/v) and the fraction is incubated at 37°C for 2

hrs. All glucosyltransferase enzymes along with other proteins that bind to dextrans or have affinity for sucrose form complexes that are removed by low speed centrifugation. The supernatant fluid is then run successively on Ultrogel AcA44 and Ultrogel AcA34. Finally, a homogeneous protein is obtained after DEAE cellulose chromatography. In E. coli, the 210 k spa A protein can be purified from the periplasmic fluid released by cold osmotic shock. Since there are few proteins of this size in the E. coli periplasm, one can obtain homogeneous preparations of this protein by ion exchange chromatography on DEAE cellulose followed by gel filtration chromatography on Ultrogel AcA34.

The spa A protein is assayed immunologically. Monospecific rabbit antibodies against the spa A protein made by both S. mutans and E. coli have been made. These antibodies have been used to measure the amount of spa A protein in the E. coli periplasm (over 80%) vs. the E. coli cytoplasm using both agar double diffusion (Fig. 5) and rocket immunoelectrophoresis. The antibody has also been conjugated against the spa A protein with rhodamine isothiocyanate to screen Streptococcal cells for the presence of spa A protein on their cell surface. This fluorescent antibody can be used to identify E. coli strains expressing the spa A protein on their cell surface.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the invention as set forth herein.

CLAIMS:

1. A vaccine suitable for the immunization of a vertebrate, which vaccine comprises a non-pathogenic microbe that expresses a recombinant gene derived from a pathogen of a vertebrate to produce an antigen capable of inducing an immune response in the vertebrate against the pathogen.

2. A vaccine as claimed in Claim 1, wherein the microbe belongs to a species that homes to a lymphoepithelial structure of the vertebrate.

3. A vaccine as claimed in Claim 2, wherein the microbe is a bacterium.

4. A vaccine suitable for the immunization of a vertebrate, which vaccine comprises an antigenic gene product of a gene derived from a first organism that is a pathogen of a vertebrate, wherein the gene was expressed in a second organism to produce the gene product and wherein the gene product is capable of inducing an immune response in the vertebrate against the pathogen.

5. A vaccine as claimed in Claim 4, wherein the vaccine is essentially free of live intact cells.

6. A vaccine as claimed in any one of Claims 1 to 5, wherein the gene codes for a surface antigen of the pathogen.

7. A vaccine as claimed in any one of Claims 1 to 6, wherein the gene codes for an enzyme of the pathogen.

8. A vaccine as claimed in any one of Claims 1 to 7, wherein the pathogen is a bacterium.

9. A vaccine as claimed in Claim 1, 2 or 3, wherein the microbe is a bacterium that belongs to the genus Salmonella or Escherichia or is Salmonella-Escherichia hybrid.

10. A vaccine as claimed in Claim 8, wherein the bacterium belongs to a species selected from the group consisting of Escherichia coli, Vibrio Chlorerae, Shigella, Pseudomonas aeruginosa, Neisseria gonorrhoeae, Chlamydia trachomatis, and Streptococcus mutans.

11. A vaccine as claimed in Claim 9, wherein the bacterium is E. coli ^{R}DEC-1 or E.coli K-12.

12. A vaccine as claimed in Claim 1 or 2, wherein the pathogen is Streptococcus mutans and the microbe is an Escherichia coli or Salmonella bacterium, or is a Salmonella-Escherichia hybrid.

13. A vaccine as claimed in Claim 12, wherein the gene codes for a surface antigen or a glycosyltransferase enzyme.

14. A vaccine as claimed in Claim 4 or 5, wherein the pathogen is Streptococcus mutans and the second organism is a bacterium.

15. A vaccine as claimed in Claim 14, wherein the gene codes for a surface antigen or a glucosyltransferase enzyme.

16. A method of producing a vaccine suitable for the immunization of a vertebrate against a pathogen, comprising the steps of selecting a gene coding for an

antigen in a pathogenic organism and inserting the gene into a carrier microbe, wherein the carrier microbe expresses the gene to produce a gene product capable of inducing antibodies against the pathogenic organism or a metabolic product of the pathogenic organism when the gene product is administered to the vertebrate.

17. The method of Claim 16, wherein the carrier microbe is a bacterium.

18. The method of Claim 17, wherein the bacterium belongs to a species that homes to a lymphoepithelial structure of the vertebrate.

19. The method of Claim 16, 17, or 18, wherein the gene codes for a surface antigen or enzyme of the pathogen.

20. The method of any one of Claims 16 to 19, wherein the bacterium belongs to the genus Salmonella or Escherichia or is a Salmonella-Escherichia hybrid.

21. The method of any one of Claims 16 to 20, wherein the pathogenic organism is Streptococcus mutans.

22. The method of any one of Claims 16 to 21, wherein the vaccine comprises the gene product in the absence of the carrier microbe.

23. The method of any one of Claims 16 to 21, wherein the carrier microbe is non-pathogenic.

24. The method of Claim 23, wherein the vaccine comprises said gene product and the carrier microbe.

25. The method of any one of Claims 16 to 24, wherein the gene is inserted into a plasmid prior to insertion into the carrier microbe.

26. A vaccine suitable for the desensitization of a vertebrate to an allergen, which vaccine comprises an antigenic gene product of a gene derived from a first organism that produces an allergen of said vertebrate, wherein said gene is expressed in a second organism which produces the gene product and wherein the gene product is an allergen of said vertebrate.

27. A vaccine suitable for the immunization of a human, which vaccine comprises a non-pathogenic microbe that expresses a recombinant gene derived from a human pathogen to produce an antigen capable of inducing an immune response in a human against the pathogen.

28. A vaccine suitable for the immunization of a human, which vaccine comprises an antigenic gene product of a gene derived from a first organism that is a human pathogen, wherein the gene was expressed in a seond organism to produce the gene product and wherein the gene product is capable of inducing an immune response in a human against the pathogen.

29. A non-pathogenic microbe that expresses a recombinant gene derived from a pathogen of a vertebrate to produce an antigen capable of inducing an immune response in the vertebrate against the pathogen.

30. A microbe according to Claim 29, wherein the vertebrate is a human.

31. An antigenic gene product of a gene derived from a first organism that is a human pathogen, wherein the gene was expressed in a second organism to produce the gene product and wherein the gene product is capable of inducing an immune response in a human against the pathogen.

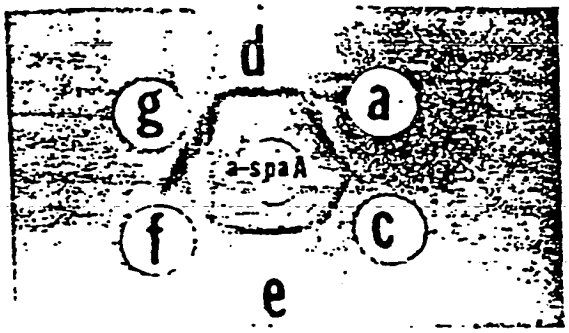

FIG. 1

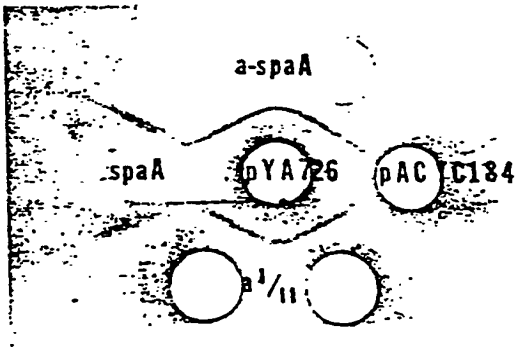

FIG. 2

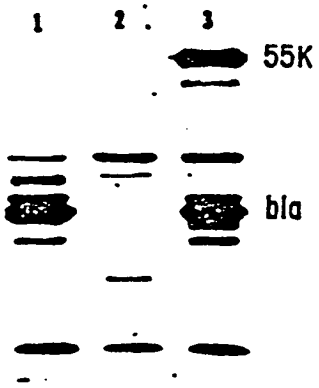

FIG. 3

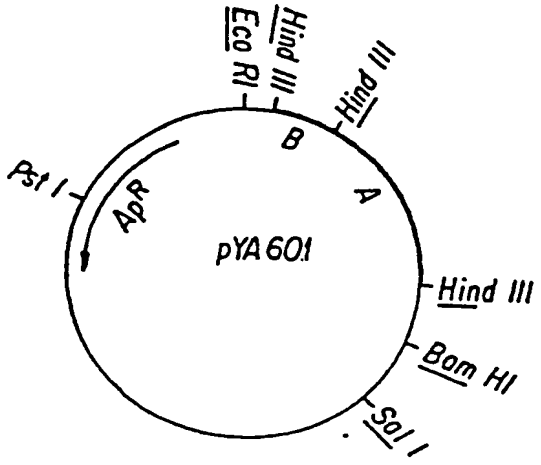

FIG. 4

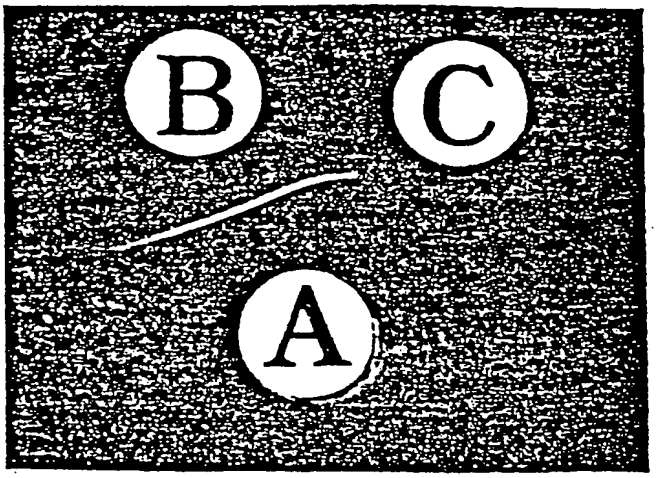

FIG. 5

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0080806
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EP 82 30 5632

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Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. ³)
X	NATURE, vol. 242, 20th April 1973, pages 531-532; J.M.RUTTER et al.: "Protection against enteric disease caused by Escherichia coli - a model for vaccination with a virulence determinant". *The whole article*	1-6,8-10,16-20,22,23,25,27-31	
X	EP-A-0 001 930 (A.D.RIGGS) *Abstract; page 11, lines 31-35; page 14, lines 1-19; page 17, lines 36-38; page 18, lines 1-21*	1-5,11,16-19,22,23,27-31	
X	FR-A-2 282 908 (M.R.WILSON) *Page 1, lines 31-39; page 2, lines 1-28,24-29; page 3, lines 1-22; claims 1,9,11*	1-4,6,8,9,15-20,23-29	TECHNICAL FIELDS SEARCHED (Int. Cl. ³) C 12 N A 61 K
X	US-A-4 237 224 (S.N.COHEN et al.:) *Abstract; column 1, lines 53-68; column 2, lines 47-51; column 16, lines 54-68; column 17, lines 1,2; claims 11-14*	7,19	

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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	05-01-1983	ENGELBRECHT E

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X	DE-A-2 530 275 (O.WESTPHAL et al.) *Page 1, paragraph 1; paragraph 2, lines 1-3; page 2, paragraphs 3,4,5; page 3, paragraph 1; page 6, paragraphs 3,4,5; page 10, paragraph 2; claims 1-8,11*	1-4,6, 8,9,16 -20,23 -25,27 -31	
Y		12	
Y	CHEMICAL ABSTRACTS, vol. 94, 1981, page 374, no. 170896e, Columbus Ohio (USA); J.B.HANSEN et al.: "Characterization of the streptococcus mutans plasmid pVA318 cloned into escherichia coli". & INFECT. IMMUN. 1981, 31(3), 1034-43. *Abstract*	12,13, 14,15, 21	TECHNICAL FIELDS SEARCHED (Int. Cl. ³)
A	GB-A-2 033 223 (G.COLMAN et al.) *Page 1, lines 3-5,40-50; page 2, lines 42-49; page 3, lines 6-63; page 4, lines 1-13,24-48*	13,15	

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A	FR-A-2 442 271 (M.HOFNUNG et al.)		
A	EP-A-0 027 662 (P.H.HOFSCHNEIDER)		
A	NATURE, vol. 238, 28th July 1972, page 219; S.J.CHALLACOMBE et al.: "Serum and salivary antibodies to glucosyltransferase in dental caries in man".		TECHNICAL FIELDS SEARCHED (Int. Cl. ³)
A	US-A-4 133 875 (J.D.HILLMAN)		

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A	CHEMICAL ABSTRACTS, vol. 88, 1978, page 240, no. 117440b, Columbus Ohio (USA); Y.KEIJI: "Application of plasmids, focusing on the genus pseudomonas. A review". & HAKKO KOGAKU KAISHI 1978, 56(1), 57-73. *Abstract*	10	
A	CHEMICAL ABSTRACTS, vol. 93, 1980, page 460, no. 65528t, Columbus Ohio (USA); M.LOVETT et al.: "Plasmids of the genus chlamydia"& CURR. CHEMOTHER. INFECT. DIS., PROC, INT. CONGR. CHEMOTHER., 11th 1979 (PUB. 1980), 2, 1250-2. *Abstract*	10	TECHNICAL FIELDS SEARCHED (Int. Cl. ³)
A	CHEMICAL ABSTRACTS, vol. 91, 1979, page 347, no. 171499c, Columbus Ohio (USA); M.C.ROBERTS : "Plasmids and conjugation in neisseria gonorrhosa". & DISS. ABSTR. INT. B 1979, 40(2), 607-8. *Abstract*	10	

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0080806
Application number

EP 82 30 5632

DOCUMENTS CONSIDERED TO BE RELEVANT

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